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Effects of chitosan and plant extracts on growth of *Colletotrichum gloeosporioides*, anthracnose levels and quality of papaya fruit

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Abstract

The objectives of this research were to evaluate the in vitro fungicidal effect of chitosan and aqueous extracts of custard apple leaves, papaya leaves and papaya seeds, and the combination of chitosan and plant extracts on the development of *Colletotrichum gloeosporioides*, which causes anthracnose on papaya. Chitosan at 2.0% and 3.0% had a fungicidal effect on *C. gloeosporioides*. Extracts alone did not show any fungicidal effect while the combination of 2.5% chitosan with all the tested extracts had a fungistatic rather than fungicidal effect. Changes in the conidial morphology of *C. gloeosporioides* were observed with 1.5% chitosan concentration after 7 h incubation. For in situ studies, control of anthracnose disease was obtained with 1.5% chitosan applied before *C. gloeosporioides* inoculation. *Phomopsis* was most frequently isolated from the non-inoculated fruit. Chitosan applications did not influence the content of total solid solubles or percentage weight loss during the storage of papaya fruit. However, there was a tendency toward greater firmness in fruit treated with the papaya seed extract alone or combined with chitosan.

Keywords: *Carica papaya*; *Colletotrichum gloeosporioides*; Papaya extract; Custard apple extract; Natural compounds

1. Introduction

In postharvest studies, chitosan has been reported to maintain the quality of fruits and vegetables, reducing respiration rates, ethylene production, and transpiration (El Ghaouth et al., 1992a, b; Li and Yu, 2000). Another important attribute of this natural compound is associated with its fungistatic or fungicidal properties against pathogens of various fruits and vegetables. Growth of important postharvest fungi such as *Alternaria alternata*, *Fusarium oxysporum*, *Rhizopus stolonifer,* and *Penicillium* spp. is inhibited on nutrient media amended with various concentrations of chitosan (Hirano and Nagao, 1989; Benhamou, 1992; Bhaskara Reddy et al., 1997; Bautista et al., 1999). In in situ studies, El Ghaouth et al. (1991; 1992a), reported a fungicidal effect of chitosan on strawberries against *Botrytis cinerea* and *R. stolonifer*. Luna et al. (2001) also reported less postharvest rots when papaya fruit were dipped in chitosan solutions compared with other postharvest treatments such as heat and thiabendazole applications.

The antimicrobial properties of plant extracts from various species have been proven to affect fungal development in vitro and in vivo (Montes-Belmont et al., 2000). Spore formation and germination, mycelial growth and infection can sometimes be stimulated or inhibited by plant extracts (Bautista-Baños et al., 2000a). In postharvest studies, dipping fruit in plant extracts inhibited rot development during storage. Among various plant species tested, aqueous extracts of leaves of papaya and custard apple (*Annona reticulata*) showed important fungistatic effects against *Rhizopus stolonifer* and *Colletotrichum gloeosporioides* in ciruela (*Spondias purpurea*) and mango (*Mangifera indica*) during fruit storage (Bautista-Baños et al., 2000b, 2002).

Postharvest diseases greatly reduce the storage life of papayas. Recently the fungus *C. gloeosporioides* has

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posed serious problems to Mexican producers because of favourable environmental conditions for this pathogen and resistance of *C. gloeosporioides* to synthetic fungicides (*personnel communication*: Rubén Mandujano).

The objectives of this work were to evaluate the effect of chitosan and aqueous extracts of custard apple leaves and papaya leaves and seeds on *C. gloeosporioides* and to study the effects of these treatments on fruit quality.

2. Materials and methods

2.1. Materials

Custard apple and papaya leaves were collected from commercial orchards located at the state of Morelos, Mexico. Papaya seeds were obtained from mature papaya fruit. For in situ experiments, papaya fruit var. Maradol at the green to yellow colour stage (index 2) were obtained from the wholesale market at Cuautla state of Morelos, Mexico. Chitosan (trade name Fluka) was obtained from BioChemika, Sigma-Aldrich, Steinham, Switzerland.

2.2. Colletotrichum gloeosporioides isolate and culture conditions

To isolate *Colletotrichum gloeosporioides* from infected papaya, fruit was placed in a humid chamber at ambient temperature (25–28°C.). Small portions of symptomatic tissue were placed on Petri plates containing Potato dextrose agar (PDA) (trade name Bioxon from Becton Dickinson, Cuatitlan Izcalli, State of Mexico, Mexico) and incubated at ambient temperature. Conidia were observed with an optical microscopy and identification was according to a published description (Barnett and Hunter, 1972). The isolate was maintained in PDA at 25–28°C. Continuous re-inoculations and re-isolations on papaya fruit were carried out to maintain pathogenicity of the inoculum.

2.3. Plant extracts and chitosan preparation

Leaves or seeds without damage or disease symptoms were selected from the collected plant material. Plant material was rinsed in sodium hypochlorite (10%) and distilled water, air-dried, macerated in a blender and stored at ambient temperature in amber bottles until use. For in vitro and in situ studies, aqueous extracts of the plant material (2:10 w/v) were left at room temperature (25–28°C) for 24 h, filtered and sterilised. For in vitro studies, before sterilisation, extracts were incorporated (10:4 v/v) into PDA. Chitosan solutions were prepared by dissolving 0.5, 1.5, 2.5 and 3.0 g of chitosan in 100 ml of distilled water with 2 ml of acetic acid, then heating with constantly agitation for 24 h. The solution was adjusted to pH 5.5 by adding sodium hydroxide 1 N, then 0.1 ml of Tween 80 was added (El Ghaouth et al., 1991).

2.4. In vitro evaluation of the fungicidal activity of chitosan and plant extracts

The effect of chitosan and/or three plant extracts on the growth of *C. gloeosporioides* was studied using agar plates. An agar disk (5 mm dm) from a pure culture of *C. gloeosporioides* was placed in the center of a PDA plate containing chitosan at 0.5, 1.5, 2.5 or 3.0%; aqueous extracts of custard apple leaves, papaya leaves, or papaya seeds (2/10 w/v) or each plant extract combined with 2.5% chitosan. Control plates contained PDA only. Petri plates incubated at 25°C for 7 d at most. Daily radial measurements of growth were taken until the fungus reached the edge of the plate. Spores were then harvested by scrapping them off the agar with the aid of a glass rod and distilled water. The mycelium and the spore mixture was double filtered through cheesecloth and resulting filtrate was adjusted to 20 ml. The number of spores/ml of filtrate was determined using a Neubauer haemocytometer.

2.5. Microscopic studies

Conidia produced by *C. gloeosporioides* growing on PDA or PDA amended with 1.5% chitosan were incubated for 7 h on water agar. The area (mm^2), length (mm) and the elliptical form of the conidia were measured before and after incubation. Images of conidia were obtained using a microscope (Nikon, Alphaphot–2 YS2) with a charged-coupled device camera (Nikon, Coolpix 900). Magnification of the image was 40×. Images were analysed using Meta Imaging series software, (version 4.0 for Microsoft Windows, Universal Imaging Corporation, USA). Forty conidia per treatment were measured.

2.6. In situ evaluation of the fungicidal activity of chitosan and aqueous extracts

The effect of chitosan (0.5% and 1.5%) and/or aqueous papaya seed extracts on growth of *C. gloeosporioides* and other postharvest fungi was evaluated in three different experiments: Papaya fruit were washed with sodium hypochlorite (1%), rinsed with distilled water and ambient air-dried. Then these fruit were artificially inoculated before or after treatments (for the first and second experiments). For the third experiment fruit were only washed with distilled water and dried. For all experiments, fruit were dipped for 20 min in (1) chitosan 0.5%, (2) chitosan 1.5%, (3) aqueous extract of papaya seed (2:10 w/v), (4) chitosan 0.5% + aqueous extract of papaya seed, (5) chitosan 1.5% + aqueous

extract of papaya seed or (6) water treatment (control). For the first and second experiments, papaya fruit were evenly sprayed with a spore suspension of *C. gloeosporioides* (1×10^6 spores/ml) and held at ambient temperature (25–28°C) for 5 days. For the third experiment, fungi were isolated and identified as previously described (Section 2.1). At the end of the storage period, disease was evaluated as incidence and severity (ranked 1–5 where 1 = 0% of fruit surface rotten, 2 = 1–25%, 3 = 26–50%, 4 = 51–75% and 5 = 76–100%) and quality was evaluated as firmness, total solid solubles (TSS) and weight loss.

2.7. Statistical analysis

For in vitro and in situ studies, treatments were arranged in a completely randomized design. Mean separation by Tukey's multiple range test ($P < 0.05$) was carried out for all parameters except for mycelial growth. To fulfil the ANOVA assumption of data from sporulation, and area and length of conidia were squared root transformed. There were three replicates for in vitro experiments. For in situ experiments, three and four replicates (experiment 1 and experiments 2 and 3, respectively) of 5 fruit each for each treatment were used. Percentage disease was analysed using the X^2 procedure.

3. Results

Mycelial growth of *Colletotrichum gloeosporioides* in vitro, was completely inhibited by chitosan concentrations of 2.5% and 3.0% during the 7 day incubation period, while at 0.5% and 1.5% concentrations, growth began on the second and fourth day of the incubation period, respectively, and ceased after 4 and 6 day, respectively (Fig. 1a). Compared to the control, none of the plant extracts tested inhibited the growth of *C. gloeosporioides*, which grew similarly with all treatments through the 7 day incubation period (Fig. 1b). For the combination of chitosan and plant extracts, growth of *C. gloeosporioides* was similar to that of the control for the first three days of incubation, then, growth suppression became evident at the end of the incubation period. Growth in the control plates was almost twice than in the treatments plates (Fig. 1c). Sporulation was significantly reduced ($P < 0.001$) by all treatments except for extracts of papaya seeds and chitosan plus custard apple leaves extract (data not shown). Sporulation was most reduced when *C. gloeosporioides* was grown on PDA amended with chitosan at 1.5% (1.8×10^6), on extracts of papaya leaves and custard apple leaves (1.8×10^6 and 1.5×10^6, respectively) and on chitosan plus papaya seed extracts (5.5×10^6).

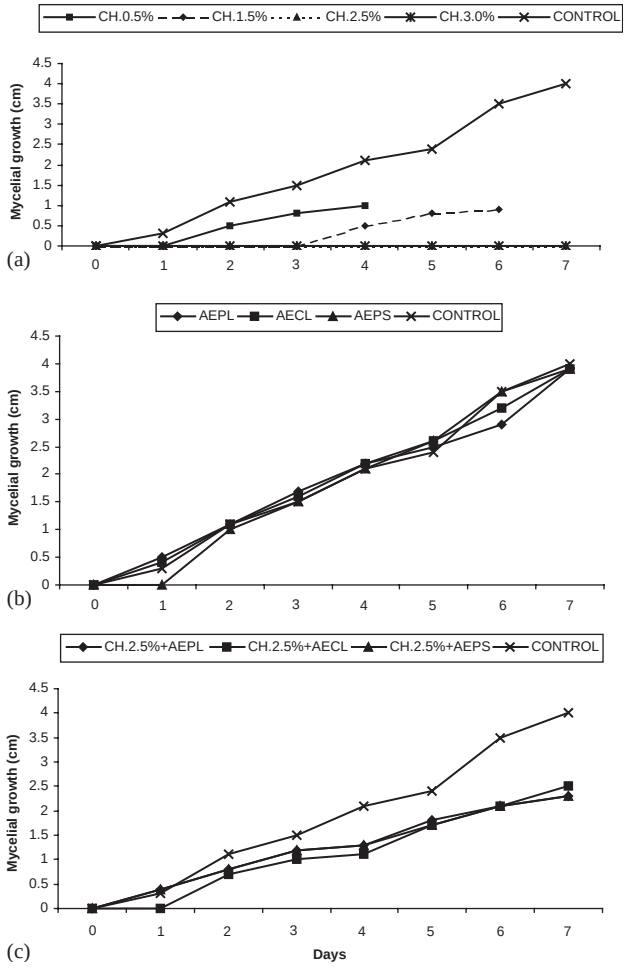

Fig. 1. (a) Effect of chitosan (CH) at various concentrations, b) aqueous extracts of papaya leaves (AEPL), custard apple leaves (AECL) and papaya seeds (AEPS) and c) combinations of chitosan and plant extracts on mycelial growth of *C. gloesporioides* during a 7d incubation period.

The area and the elliptical form of spores were significantly different from the control ($P < 0.001$) when *C. gloeosporioides* was grown on PDA amended with 1.5% chitosan. For the main effect time, area, length, and form were significantly higher ($P < 0.001$) after 7 h incubation (Table 1). No significant differences were observed for the interaction of treatment and time.

There were significant differences among treatments ($P < 0.001$) in fruit inoculated after treatment application in the in situ experiments (Tables 2 and 3). The highest fungicidal effect was observed in those papayas treated with 1.5% chitosan alone or with the combination of 1.5% chitosan and AEPS (incidence of 40% and 47%, respectively) and disease severity index of 1.4 and 1.5 indicating fruit surface infection close to 1% ($P < 0.001$). When fruit were inoculated before treatment no significant differences were observed. Incidence was high in all treatments (95–100%). Disease severity index

varied from 3 to 5 (26–100% of the fruit surface infected) ($P<0.001$). In the non-inoculated fruit, there were significant differences among treatments ($P<0.01$). Papaya fruit treated with 0.5% or 1.5% chitosan or the combination of 1.5% chitosan with AEPS had the least infection (60%), while the lowest disease severity index obtained was for fruit treated with 1.5% chitosan ($P<0.001$).

Table 1
Effect on area, length and elliptical form of spores of *C. gloeosporioides* growing on PDA amended with chitosan at 1.5%

Source	Level	Area[a,b] (mm^2)	Length[a,b] (mm)	Elliptical form[b]
Main effect				
Treatment		$P<0.001$	NS	$P<0.001$
	Control	4.2b	2.9a	1.7b
	Chitosan 1.5%	5.2a	3.0a	1.9a
Time[c](h)		$P<0.001$	$P<0.001$	$P<0.001$
	0	4.3b	2.7b	1.6b
	7	5.2a	3.3a	2.0a
Treat *x* time		NS[d]	NS[d]	NS[d]

[a] *P* values after square root transformation.
[b] Average of 40 observations.
[c] period of incubation at 25°C on water agar.
[d] NS = non-significant.

Among fruit inoculated before or after treatments, the highest TSS values ($P<0.05$ and $P<0.001$, respectively) were recorded in the control fruit, while for the non-inoculated fruit the highest values were for fruit treated with 0.5% chitosan (Table 2). There were significant differences ($P<0.01$) in firmness among treatments in the three experiments. Firmness value was highest in fruit treated with chitosan and AEPS except when the inoculation occurred after treatment. No differences were observed in percentage mass loss among treatments (Table 2). For the experiments with non-inoculated papaya fruit, the main pathogen isolated and identified after the storage period was *Phomopsis* (data not shown).

4. Discussion

Chitosan alone had the greatest effect against *Colletotrichum gloeosporioides* in both in vitro and in situ experiments. *Colletotrichum gloeosporioides* is very sensitive to chitosan since growth was affected at all concentrations including only 0.5%. Mycelial growth, sporulation and conidial morphology were affected by chitosan indicating that chitosan affected various stages of the development of *C. gloeosporioides*. Mycelial growth and spore formation of *Rhizopus stolonifer* and

Table 2
Effect of chitosan and aqueous extract of papaya seed (AEPS) on total solid solubles (TSS), firmness, mass loss, disease incidence and severity for papayas inoculated with *C. gloeosporioides* or non-inoculated and stored at ambient temperature

Treatments	TSS (%)[a,b]	Firmness (N)[a,c]	Mass loss (%)[a]	Incidence (%)[a]	Severity index[a]
Inoculation after treatments	$P<0.05$	$P<0.01$	NS	$P<0.001$	$P<0.001$
1. Chitosan 0.5%	9.3ab	11.0b	7.9a	73a	1.7c
2. Chitosan 1.5%	8.6ab	12.0b	7.4a	40b	1.4d
3. AEPS	7.9b	24.9a	7.4a	100a	5.0a
4. Chitosan 0.5% + AEPS	8.7ab	12.0b	8.9a	80a	2.6b
5. Chitosan 1.5% + AEPS	8.6ab	10.0b	8.1a	47b	1.5d
6. Control	9.6a	3.0c	7.4a	93a	2.8b
Inoculation before treatments	$P<0.01$	$P<0.01$	NS	NS	$P<0.001$
1. Chitosan 0.5%	8.5ab	25.0c	7.9a	100a	4ab
2. Chitosan 1.5%	8.1ab	26.0c	8.7a	100a	5a
3. AEPS	7.6b	23.0b	8.7a	100a	5a
4. Chitosan 0.5% + AEPS	8.3ab	35.0a	7.6a	100a	4ab
5. Chitosan 1.5% + AEPS	8.5ab	36.0a	7.4a	95a	4ab
6. Control	8.9a	25.0c	7.3a	95a	3b
Non-inoculated	NS	$P<0.01$	NS	$P<0.01$	$P<0.001$
1. Chitosan 0.5%	9.4a	29.0b	5.5a	60b	1.9bc
2. Chitosan 1.5%	8.9a	25.0b	5.4a	60b	1.8d
3. AEPS	8.7a	29.0b	5.8a	70ab	2.0bc
4. Chitosan 0.5% + AEPS	8.6a	40.0a	5.7a	75a	2.1ab
5. Chitosan 1.5% + AEPS	8.4a	42.0a	5.7a	60b	2.0bc
6. Control	8.9a	26.0b	5.5a	75a	2.2a

Severity index: 1 = 0%, 2 = 1–25%, 3 = 26–50%, 4 = 52–75%, 5 = 76–100%.
[a] Means separation within each experiment by Tukey's multiple test ($P<0.05$).
[b] Initial TSS for the first, second and third experiment = 8.7%, 8.5% and 8.9%, respectively.
[c] Initial firmness for the first, second and third experiment = 43, 42 and 45 N, respectively.

Table 3
Identification of fungi isolated from non-inoculated papayas that were treated with chitosan plus aqueous extract of papaya seeds and stored at ambient temperature for 5 days

Treatments	Microorganism	Percent of infected fruit
1. Chitosan 0.5%	*Aspergillus*	5
	Colletotrichum	15
	Fusarium	10
	Phomopsis	75
2. Chitosan 1.5%	*Aspergillus*	5
	Colletotrichum	15
	Fusarium	15
	Phomopsis	60
3. AEPS	*Aspergillus*	5
	Phomospsis	60
4. Chitosan 0.5% + AEPS	*Phomopsis*	70
	Rhizopus	5
5. Chitosan 1.5% + AEPS	*Colletotrichum*	5
	Fusarium	10
	Phomopsis	55
6. Control	*Fusarium*	10
	Phomopsis	75
	Rhizopus	10

Botrytis cinerea are reduced as the chitosan concentration increases (El Ghaouth et al., 1992a). Similarly, serious alterations of the morphology of the mycelium of various other pathogenic microorganisms have been reported when grown on chitosan (Benhamou, 1992; Benhamou et al., 1994; El Ghaouth et al., 1997). In those studies severe cellular damage was observed of the mycelium of the fungi treated with chitosan. Additional work is needed to determine if similar cellular changes occur in *C. gloeosporioides*.

Chitosan has a protective effect on papaya fruit, rather than a therapeutic effect since chitosan was more effective when applied before *C. gloeosporioides* inoculation than when applied afterwards or applied to naturally infected fruit. Bashkara Reddy et al. (2000) report similar results when applications of chitosan were applied to strawberry plants before artificial inoculation. It is possible that chitosan coating on papaya fruit acted as a barrier limiting penetration of the germ tube of *C. gloeosporioides*. However, other physiological events might be involved in control of anthracnose during papaya storage since chitosan has also been mentioned to induce several host defense mechanisms such as the induction of physical barriers and production of phytoalexins (Hadwiger and Beckman, 1980; El Ghaouth et al., 1994).

AEPS only affected sporulation of *C. gloeosporioides*, lacking the fungistatic or fungicidal effect reported in our previous experiments (Bautista-Baños et al., 2000b) when applied to yellow 'ciruelas' to control *R. stolonifer* during storage. In other studies we have observed similar selective fungistatic effect from plant extracts. We have found differences in the fungicidal activity of extracts from the same plant species associated with various factors such as solvent used, plant organ, harvesting season and climatic conditions (Bautista-Baños et al., 2002, 2003). In the present study, no synergistic effect was obtained with the combination of chitosan at 1.5% and AEPS to control *C. gloeosporioides*.

In the non-inoculated experiment, the fungus most frequently isolated from naturally infected papaya fruit was *Phomopsis*. It was not isolated from fruit inoculated with *C. gloeosporioides* before or after treatments. Although fruit was obtained from the same wholesale market it might be that the production location was different. *C. gloeosporioides* is the main pathogen of papaya reported in the papaya production areas of Mexico. However, *Phomopsis* has also been mentioned to be a serious postharvest disease of papaya with latency behaviour (Alvarez and Nishijima, 1987; Snowdon, 1990).

Firmness was the physiological parameter most affected by the treatments. Although there was not a consistent pattern among the three experiments conducted, we could observe the highest firmness in fruit treated with AEPS or treated with chitosan combined with this extract. Previous studies report greater firmness of fruit such as strawberries, tomatoes and peaches when coated with a chitosan solution. (El Ghaouth, 1991; 1992b; Li and Yu, 2000; Luna et al., 2001). In the present study, fruit treated with chitosan alone did not have the greatest firmness, despite better disease control provided by chitosan than AEPS, suggesting that fruit in AEPS delayed papaya fruit ripening hence resulting in firmer fruit. Studies carried out by Mosch et al. (1993) reported that plant extracts besides controlling pathogen disease, induce several host defense mechanisms such as certain enzymatic activity.

We believe that 60% control of anthracnose on papaya fruit achieved with chitosan is sufficiently adequate to consider chitosan as a natural product to control postharvest diseases of papaya. However, because of the quiescence infection of *C. gloeosporioides,* additional work is needed to evaluate field applications of chitosan.

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